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Prominin-2 expression increases protrusions, decreases caveolae and inhibits Cdc42 dependent fluid phase endocytosis

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Abstract

Background—Membrane protrusions play important roles in biological processes such as cell adhesion, wound healing, migration, and sensing of the external environment. Cell protrusions are subtype of membrane microdomains composed of cholesterol and sphingolipids, and can be disrupted by cholesterol depletion. Prominins are pentaspan membrane proteins that bind cholesterol and localize to plasma membrane (PM) protrusions. Prominin-1 is of great interest as a marker for stem and cancer cells, while Prominin-2 (Prom2) is reportedly restricted to epithelial cells.

Aim—To characterize the effects of Prom-2 expression on PM microdomain organization.

Methods—Prom2-fluorescent protein was transfected in human skin fibroblasts (HSF) and Chinese hamster ovary (CHO) cells for PM raft and endocytic studies. Caveolae at PM were visualized using transmission electron microscopy. Cdc42 activation was measured and caveolin-1 knockdown was performed using siRNAs.

Results—Prom2 expression in HSF and CHO cells caused extensive Prom2-positive protrusions that co-localized with lipid raft markers. Prom2 expression significantly decreased caveolae at the PM, reduced caveolar endocytosis and increased caveolin-1 phosphorylation. Prom2 expression also inhibited Cdc42-dependent fluid phase endocytosis *via* decreased Cdc42 activation. Effects on endocytosis were reversed by addition of cholesterol. Knockdown of caveolin-1 by siRNA restored Cdc42 dependent fluid phase endocytosis in Prom2-expressing cells.

Conclusion—Prom2 protrusions primarily localize to lipid rafts and recruit cholesterol into protrusions and away from caveolae, leading to increased phosphorylation of caveolin-1, which inhibits Cdc42-dependent endocytosis. This study provides a new insight for the role for prominins in the regulation of PM lipid organization.

Keywords

lipid rafts; filopodia; sphingolipids; Rho proteins

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INTRODUCTION

Membrane protrusions play important roles in biological processes such as cell adhesion, wound healing, migration, and sensing of the external environment [1,2]. Several types of plasma membrane (PM) protrusions exist, such as lamellipodia, sheet-like extensions of the cell supported by branched actin filaments, and filopodia, finger-like projections supported by parallel actin bundles [3,4]. Cell protrusions have been proposed to be a type of membrane microdomain, as they possess elevated levels of cholesterol and glycosphingolipids (GSLs), relative to other regions of the cell membrane [5,6,7] and protrusion structure can be disrupted by cholesterol depletion [8,9,10]

Prominin proteins (Prom1 and Prom2) are pentaspan transmembrane proteins that are enriched at PM protrusions in some cell types [5,11,12]. Both prominins have been shown to directly bind cholesterol and associate with membrane microdomains in living cells [13,14,15]. Prom1 (CD133) has been widely studied as a marker for certain stem cells and cancer stem cells [5,12,16], whereas Prom2 has been shown to be present in some epithelial cells but is otherwise little studied [11,17,18]. The prominins have been proposed to be involved in the organization of membrane protrusions but the specific function of these proteins is presently unknown [5,11].

Our laboratory has been interested in the function and distribution of microdomains on the PM of living cells, and has used various fluorescent probes such as BODIPY-lactosylceramide (Bodipy-LacCer), polyethylene glycol-coupled cholesterol (PEG-Chol), and cholera toxin B subunit (CtxB) to label such domains [19,20,21]. Since cell protrusions have been reported to be a type of GSL-enriched microdomain [7,22], we over-expressed Prom2 as a marker for protrusions and investigated the colocalization of this protein with Bodipy-LacCer and other lipid raft markers. The fluorescent lipid, along with other lipid raft markers, was found to be highly co-localized with Prom2 in protrusions. Over-expression of Prom2 led to significant changes in PM organization and function, including increased protrusions, decreased caveolae at the PM, and decreased caveolar and fluid phase endocytosis. It also resulted in increased caveolin-1 phosphorylation, which inhibited Cdc42-dependent endocytosis due to Cdc42 inactivation. This study provides new insight into possible roles of prominin proteins in regulating PM organization.

MATERIALS AND METHODS

Cell Culture

Normal human skin fibroblasts (HSFs; GM-5659, Coriell Institute for Medical research, Camden, NJ) and CHO cells (ATCC, Manassas, VA) were grown as described [20].

Constructs and transfection experiments

A DNA construct encoding full length human Prom2 was purchased (Thermo Scientific, Waltham MA) and modified (see Suppl). Transfection of DNA constructs was performed using a Nucleofector II apparatus (Lonza).

Lipids, Fluorescent Probes and Miscellaneous Reagents

Bodipy-D-*e*-LacCer was complexed to defatted BSA as described [23]. Fluorescent AF488-Tfn, AF488-dextran (10kD), and secondary antibodies were from Invitrogen (Eugene, OR). Rhodamine-wheat germ agglutinin (Rh-WGA) was from Vector Laboratories (Burlingame, CA). PEG-Chol (a kind gift from Toshihide Kobayashi, Riken) was labeled with AF488 carboxylic acid (Invitrogen). Caveolin-1 (Cav1) and pY14-Cav1 antibodies were from BD Biosciences (San Jose, CA). A Cdc42 activation kit and HRP-conjugated secondary

antibodies were from Millipore (Billerica, MA). All other reagents were from Sigma-Aldrich.

PM labeling with fluorescent probes

HSFs were transfected with Prom2-GFP or -mKate for 48 h. Transfected HSFs were then washed with ice cold HMEM and incubated with 5 μg/ml Rh-WGA, 2.5μM Bodipy-LacCer, 2 μg/ml AF488-CtxB or AF594-StxB, or AF488-PEG-Chol for 30 min at 10°C to label the PM. Samples were then washed, and images were acquired at appropriate wavelengths for the different fluorophores.

Endocytosis assays

For endocytosis assays, HSFs were transfected for 48 h with Prom2 - mKate or –GFP and endocytosis assays were performed as described [20]. In some experiments 5mM methyl ß-cyclodextrin/cholesterol (MßCD/Chol) complex (Sigma) was added to cells for 30 min at 37°C prior to endocytosis assays.

Electron microscopy

Electron microscopy studies were carried out in the Mayo Clinic Electron Microscopy Core Facility (see Suppl.) using FEI Tecnai T12 transmission electron microscope and Hitachi S-4700 cold field-emission electron microscope for transmission and scanning electron microscopy, respectively.

Fluorescence Microscopy and Analysis

Olympus IX70 fluorescence microscope equipped with 60× 1.4NA or 100× 1.35 NA oil immersion objectives was used for fluorescence microscopy. Images were acquired using a Quant EM:512SC CCD camera (Photometrics, Tucson AZ). For quantitation assays, all photomicrographs in a given experiment were exposed and processed identically for a given fluorophore and were analyzed using MetaMorph image processing program (version 7.3.2; Universal imaging Corp.). Quantitative results are expressed as mean ± SE. Images were prepared for individual figures using Photoshop CS (Adobe Systems Inc., San Jose CA). No deconvolution, 3D reconstructions, surface or volume rendering, or gamma adjustments were performed on images.

Miscellaneous procedures

Cells were lysed in RIPA buffer (pH 8.0) with Complete protease inhibitor cocktail (Roche, Indianapolis IN) and Halt phosphatase inhibitor cocktail (Thermo Scientific) for SDS-PAGE and immunoblotting. HSFs were transfected with 100 nM siRNA against human Cav1 or negative siRNA using DharmaFECT 2 (all from Thermo Scientific). Immunofluorescence of formaldehyde-fixed cells was performed as described previously [20,24].

RESULTS

Characterization of membrane protrusions induced by ectopic expression of Prominin-2

HSFs were transfected with fluorescent Prom2, a marker for protrusions [11,13]. Ectopic expression of Prom2-fluorescent protein induced an extensive network of filopodia-like protrusions projecting from the cell surface (Fig. 1A). A similar induction of membrane protrusions was found when Prom2 was expressed in CHO cells (Fig. 1B). Prom2-positive protrusions were also stained by Rh-WGA (Fig. 1A,B). In contrast, few Rh-WGA-positive protrusions were observed on non-transfected cells (Fig. 1B). To confirm that expression of Prom2 fluorescent protein causes a change in morphology of the cell surface, we performed scanning electron microscopy (SEM) of transfected cells. An extensive network of

protrusions was observed in Prom2-transfected cells, which was absent in mKate control transfected cells (Fig. 1C).

We next characterized the Prom2-labeled protrusions by co-localization studies using different microdomain/lipid raft markers [19,20,21,25]. HSFs transfected with Prom2-mKate or - GFP (as indicated) were incubated with indicated markers for 30 min at 10°C, and live cell images were acquired at 10°C to inhibit endocytosis. We found an extensive overlap of protrusions with Bodipy-LacCer (Fig. 1D), AF647 labeled PEG-chol and CtxB (binds endogenous GM_1 ganglioside) (Suppl. Fig. 1), suggesting that Prom2 protrusions constitute a type of membrane domain. Fluorescent Shiga toxin B subunit (StxB; binds endogenous globoside)-enriched regions showed little overlap with Prom2 protrusions (Suppl. Fig. 1), demonstrating the presence of lipid domains that are distinct from Prom2 protrusions.

Prom2 expression induced a reduction of caveolar endocytosis and caveolae at PM

We previously found that Bodipy-LacCer is internalized primarily via caveolae in multiple cell types [20,26,27,28]. Since we demonstrated here that Bodipy-LacCer is extensively localized to Prom2-positive protrusions, we investigated whether Prom2 expression had any effect on endocytosis of Bodipy-LacCer. Uptake of Bodipy-LacCer was significantly (~50%) inhibited in Prom2-expressing cells (Fig. 2A,B). Since Prom2 has been shown to bind cholesterol [13] that is needed for the assembly and function of caveolae [29,30,31], we speculated that the formation of extensive Prom2-positive protrusions might inhibit endocytosis via caveolae by drawing cholesterol into protrusions and away from caveolae. Thus, we added exogenous cholesterol to Prom2-expressing cells and assayed Bodipy-LacCer uptake. We found that treatment of Prom2-positive cells with cholesterol restored Bodipy-LacCer endocytosis (similar to control cells) (Fig. 2B).

To further elucidate the mechanism by which Prom2 expression inhibits Bodipy-LacCer endocytosis, we examined the effect of Prom2 expression on PM caveolae using transmission electron microscopy (TEM). For these studies, HSFs were transfected with mKate (control) or Prom2-mKate and plated on gridded glass cover slips. Transfected cells were identified and located on grid using fluorescence microscopy. Cells were then fixed, processed using ruthenium red to identify cell surface-connected caveolae [32]. The number of caveolae (ruthenium red positive, uncoated surface vesicles; 50–80 nm in diameter), were counted in a blinded manner. HSFs transfected with mKate only (controls) showed numerous caveolae attached to the PM, whereas HSFs transfected with Prom2-mKate had significantly reduced PM caveolae (Fig. 2C). Quantitation of TEM images demonstrated a ~75% reduction in surface-connected caveolae in Prom2-expressing cells vs. controls (Fig. 2D).

Prom2 expression reduced Cdc42-dependent fluid phase endocytosis, Cdc42 activation and stimulated Cav1 phosphorylation

We next studied the effect of Prom2 over-expression on clathrin-independent Cdc42-dependent fluid phase endocytosis [26,33]. Surprisingly, fluid phase uptake of dextran was inhibited (>60%) in Prom2-positive cells (Fig. 3A, C), with no effect on the clathrin-mediated uptake of transferrin (Suppl. Fig. 2). Since cholesterol addition restored Bodipy-LacCer internalization (Fig. 2A), we next tested the possibility that addition of cholesterol might restore fluid phase endocytosis in Prom2-expressing cells. We found that dextran uptake in Prom2-expressing cells was restored to control levels upon cholesterol addition (Fig. 3A,B). We previously reported that fluid phase endocytosis, a Cdc42-dependent process [26,33], is inhibited under conditions where levels of Y14-phosphorylated-Cav1 (Y14-pCav1) are increased [34]. We proposed that this inhibition is due to the ability of

Cav1 to bind to Cdc42 in its inactive, GDP-bound state [35], an interaction which is enhanced by Y14-pCav1 [34]. Thus, we tried to determine if the inhibition of Cdc42-dependent endocytosis occurs by a similar mechanism in Prom2-expressing cells. We found that active Cdc42-GTP was decreased (~45%) in Prom2-GFP-expressing cells compared to GFP-expressing control cells (Fig. 3C). We also found that Y14-pCav1 levels were significantly higher (~2.5-fold) in Prom2-mKate-expressing cells compared to mKate (control)-transfected cells with no effect on total Cav1 and actin levels (Fig. 3D,E). These findings support the concept that Prom2 expression increases Cav1 phosphorylation, thus inhibiting Cdc42-dependent endocytosis.

Knockdown of Cav1 restores Cdc42 dependent fluid phase endocytosis

To confirm that the effects of Prom2 on fluid phase endocytosis are due to alterations in Cav1, we used siRNA approach to deplete Cav1 by ~95% relative to control levels (Fig. 4A), and then examined the effects of Prom2-mKate expression on dextran uptake. In cells depleted of Cav1, dextran uptake was unaffected by Prom2 expression, whereas in control cells Prom2 inhibited dextran uptake (Fig. 4B,C), similar to results seen in Fig. 3.

DISCUSSION

In the present study, we found that ectopic expression of Prom2 induced an extensive network of membrane protrusions that could be visualized by fluorescence microscopy as well as by SEM. Prom2 expression also decreased the caveolar endocytosis and surface-connected caveolae. A significant inhibition of dextran uptake by the fluid phase pathway was also observed in Prom2-expressing cells, which we propose is a result of increased Cav1 phosphorylation that inhibits Cdc42 activation. Endocytosis via caveolae and dextran uptake could be restored to normal levels by MßCD-cholesterol treatment, a finding which suggests that the effects of Prom2 expression are a result of the redistribution of limiting amounts of cholesterol away from certain domains and into protrusions. Together, these results suggest that Prom2 expression causes a redistribution of PM cholesterol to protrusions, leading to reduced cholesterol at caveolae and inhibiting fluid phase endocytosis as a result of increased Y14-pCav1/Cdc42 interaction. Our findings suggest a novel role for prominins in the organization of PM lipid microdomains and may have significance for the functions of these proteins in epithelia, cancer cells and stem cells.

Expression of Prom2 caused a loss of caveolae from the PM due to recruitment of cholesterol from PM to protrusions as determined by TEM, similar to two different reports in 3T3-L1 adipocytes, where cholesterol depletion caused the loss/flattening of caveolae [31,36]. We hypothesized that Prom expression results in a redistribution of cholesterol away from caveolae membranes, leading to the flattening/loss of PM caveolae. This premise is supported by experiments showing that the reduction of Bodipy-LacCer endocytosis by Prom expression can be reversed by incubation of cells with cholesterol (Fig. 2B). In addition, cholesterol depletion is reported to result in increased Y14-pCav1 [30]. Similarly, we found that Prom2 expression resulted in an increase in Y14-pCav1 (Fig. 3), a result consistent with the idea that the effects of Prom2 expression on Cav1 may be mediated by cholesterol redistribution.

The finding that Prom2 expression reduced PM caveolae and the endocytosis of dextran led us to consider whether these two observations are linked. Cav1, the key protein in caveolae biogenesis, was reported to act as a guanine nucleotide dissociation inhibitor of Cdc42, thus inhibiting Cdc42-dependent secretion [35]. We previously reported that fluid phase endocytosis was inhibited by Cav1 over-expression and stimulated by Cav1 depletion by siRNA, and provided evidence that this is a result of the ability of Y14-pCav1 to inhibit Cdc42 activation [34]. Here, we found that Prom2 expression caused a reduction in Cdc42

activation and dramatic increase in pCav1 levels (Fig. 3). These finding led to the hypothesis that increased pCav1 is responsible for inhibition of fluid phase uptake in Prom2 over-expressing cells. The lack of effect of Prom2 expression on fluid phase uptake in cells depleted of Cav1 (Fig. 4B,C) supports this hypothesis. Our results are consistent with a model in which Prom expression causes local losses of cholesterol in caveolar membranes resulting in increased Y14-pCav1. Higher levels of pCav1 interact with Cdc42, leading to retention of Cdc42 in its inactive state and decreasing fluid phase endocytosis.

Based on the localization of prominin proteins to cellular protrusions, they have been hypothesized to play a role in the membrane organization [5,13,16]. Further, given the ability of Prom1 and Prom2 to interact with cholesterol, it has been proposed that prominins function to regulate lipid composition in protrusions [5,13]. Our study shows that prominin protein expression has the potential to reorganize PM domains such as those associated with caveolae, in addition to promoting the formation of cell protrusions. This membrane reorganizing function may be one mechanism by which prominins affect cell differentiation and migration. Y14-pCav1 has been reported to be important for the regulation of migration [37,38,39]. Our studies suggest that prominin expression in stem cells and cancer cells might influence migration by altering the phosphorylation of Cav1. In conclusion, our findings suggest a role for prominin proteins in regulating PM lipid rafts, a concept that may aid in our understanding of the significance of prominin expression in different cell types.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

1. Prominin-2 expression induced protrusions that co-localized with lipid raft markers
2. Prominin-2 expression decreased caveolae, caveolar endocytosis and increased pCav1
3. Prominin-2 expression inhibited fluid phase endocytosis by inactivation of Cdc42
4. These endocytic effects can be reversed by adding exogenous cholesterol
5. Caveolin1 knockdown restored fluid phase endocytosis in Prominin2 expressing cells

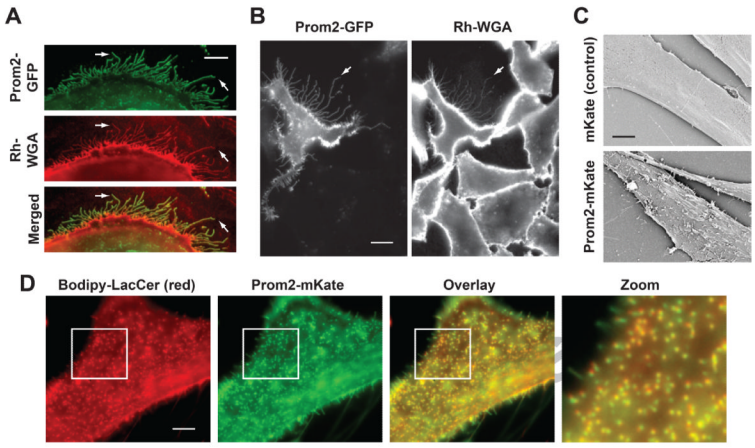

Fig. 1. Prom2 expression induced extensive protrusions that colocalize with a lipid raft marker

HSFs (A) or CHO cells (B) were transfected with Prom2 GFP (48 hr) and stained with Rh-WGA to label cell surface carbohydrates. Note the extensive protrusions labeled with both Prom2-GFP and Rh-WGA (e.g., at arrows) Bar, 10 μm. (C) HSFs were transfected with Prom2-mKate or mKate only and SEM was performed on fixed cells. Transfected cells showed extensive protrusions on their surface whereas control cells had very few protrusions. Bar, 5 μm. (D) Cells transfected with Prom2-mKate, were incubated with Bodipy-LacCer and fluorescence images of living cells were acquired for Prom-2-mKate and Bodipy-LacCer and merged. Note the extensive co-localization of Prom2 with Bodipy-LacCer. Imaging at low temperature inhibited endocytosis.

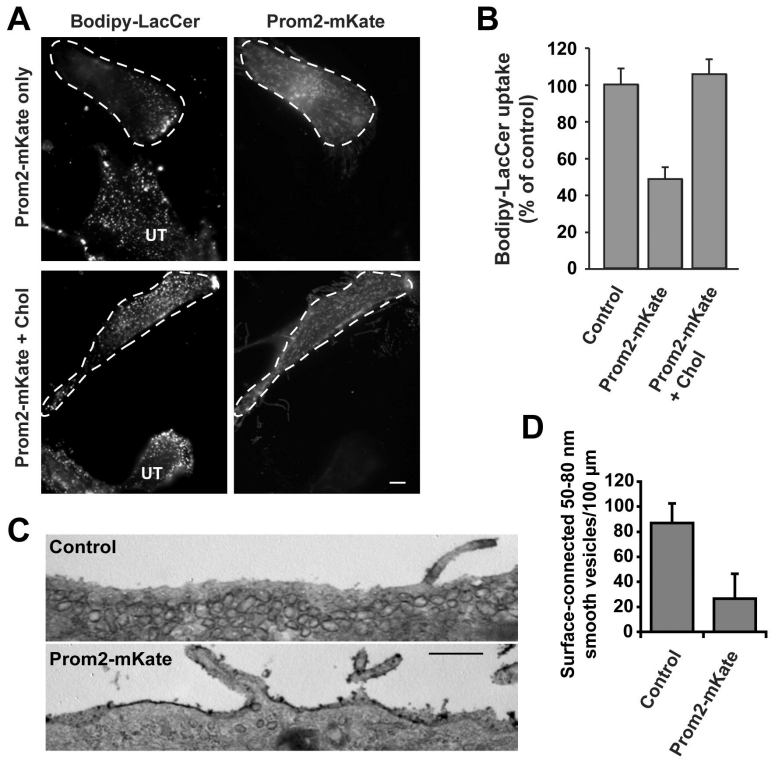

Fig. 2. Expression of Prom2 reduced caveolar endocytosis and the numbers of cell-surface attached caveolae

(A) HSFs transfected ± Prom2-mKate (48 h) were assayed for Bodipy-LacCer endocytosis as described [27]. Transfected cells are outlined with dashed lines (UT-untransfected cells). Bar, 10 μm. (B) HSFs transfected ± Prom2-mKate were quantified for Bodipy-LacCer endocytosis by image analysis. One set of Prom2-expressing cells were pre-treated with 5 mM MßCD/Chol for 30 min at 37°C before Bodipy-LacCer endocytosis. Values are expressed as percentage of control (non-transfected cells in the same field) and are mean ± SE (n>30 cells from three independent experiments). (C) HSFs, transfected with Prom2-mKate or mKate alone (controls), were cultured on gridded glass cover slips for 48 h. Transfected cells (identified by fluorescence microscopy), were stained and processed by TEM to identify PM connected caveolae. Bar, 500 nm. (D) Ruthenium-red positive smooth vesicles (50–80 nm diameters) in TEM images were counted blindly. Values are expressed as means ± SE number/100 μm of length (n>20 cells/condition from two different experiments).

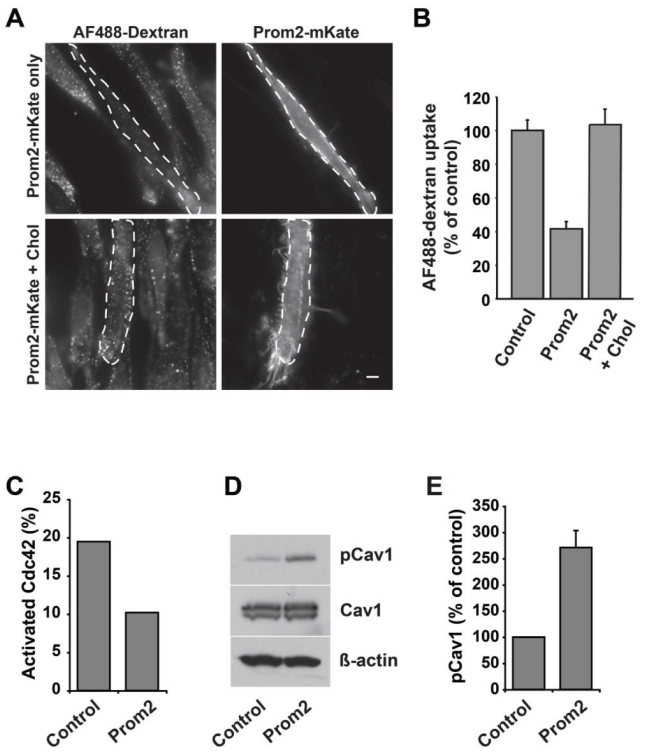

Fig. 3. Prom2 expression decreased fluid-phase endocytosis, decreased Cdc42 activation and stimulated Y14-phosphorylation of caveolin-1

HSFs transfected ± Prom2-mKate (48 h) were assayed for dextran (fluid phase uptake) endocytosis. In some cases transfected cells were pretreated with 5mM MßCD/Chol for 30 min at 37°C before endocytosis. (A) Fluorescence images of endocytosis of dextran. Transfected cells are outlined by dashed lines. Bars, 10 μm. Dextran endocytosis was quantified (B) by image analysis as in Fig. 2B. Values are means ± SE (n >30 cells in three independent experiments). Note the inhibition of dextran uptake in Prom2-transfected cells and restoration of dextran endocytosis in Prom2-transfected cells by cholesterol. (C) Cdc42 activation assay was performed in cell lysates from transfected and non-transfected cells. Activated Cdc42 (Cdc42-GTP) and total Cdc42 were immunoblotted and levels of activated Cdc42 were normalized to total Cdc42. Note the decreased activation of Cdc42 in Prom2-transfected cells. (D) Cell lysates (as above) were immunoblotted for Y14-pCav1, Cav1 and actin. Note the increased Cav1 phosphorylation in Prom2-transfected cells compared to mkate alone transfected cells. (E) Quantitation of Y14-pCav1 immunoblots. Values are means ± SE (control 100%) for three different experiments.

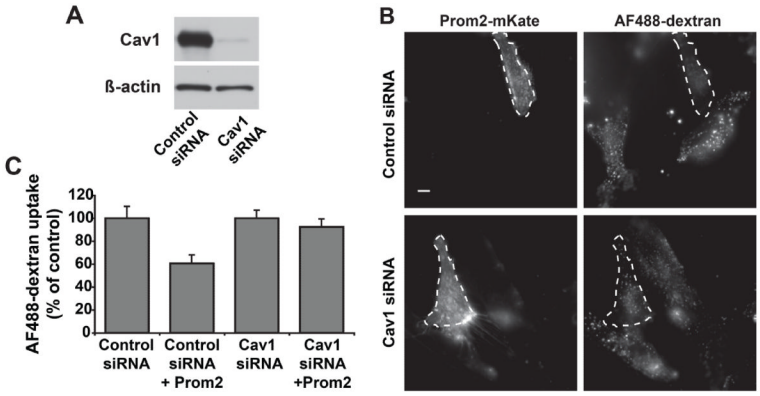

Fig. 4. Knockdown of caveolin-1 restored fluid phase endocytosis in Prom2 expressing cells

HSFs were treated with Cav1 or negative siRNA for 5 days. (A) Immunoblot showed knockdown of Cav1 in cells treated with Cav1 siRNAs. Equal amounts of protein were loaded (actin immunoblot). (B) Cells treated with Cav1 or negative siRNA were transfected with Prom2-mKate for 48 h and then AF488-dextran uptake was studied as in Fig3. Prom2-transfected cells are outlined. Bar, 10 μm. (C) Quantitation of dextran uptake in Cav1 vs. negative siRNA-treated cells ± Prom2-mKate expression as in Fig3A. Prom2-transfected cells are outlined with dashed lines. Note the reduced level of dextran uptake in negative siRNA/Prom2-expressing cells compared to untransfected cells, but the lack of effect of Prom2 expression on dextran uptake in Cav1 siRNA-treated cells.